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Phosphorylated and ubiquitinated TDP-43 pathological inclusions in ALS and FTLD-U are recapitulated in SH-SY5Y cells

Takashi Nonaka [a,*], Tetsuaki Arai [b], Emanuele Buratti [c], Francisco E. Baralle [c], Haruhiko Akiyama [b], Masato Hasegawa [a,*]

[a] Department of Molecular Neurobiology, Tokyo Institute of Psychiatry, Tokyo Metropolitan Organization for Medical Research, 2-1-8 Kamikitazawa, Setagaya-ku 156-8585, Tokyo, Japan
[b] Department of Psychogeriatrics, Tokyo Institute of Psychiatry, Tokyo Metropolitan Organization for Medical Research, 2-1-8 Kamikitazawa, Setagaya-ku 156-8585, Tokyo, Japan
[c] International Centre for Genetic Engineering and Biotechnology, 34012 Trieste, Italy

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ABSTRACT

We report phosphorylated and ubiquitinated aggregates of TAR DNA binding protein of 43 kDa (TDP-43) in SH-SY5Y cells similar to those in brains of amyotrophic lateral sclerosis (ALS) and frontotemporal lobar degeneration with ubiquitinated inclusions (FTLD-U). Two candidate sequences for the nuclear localization signal were examined. Deletion of residues 78–84 resulted in cytoplasmic localization of TDP-43, whereas the mutant lacking residues 187–192 localized in nuclei, forming unique dot-like structures. Proteasome inhibition caused these to assemble into phosphorylated and ubiquitinated TDP-43 aggregates. The deletion mutants lacked the exon skipping activity of cystic fibrosis transmembrane conductance regulator (CFTR) exon 9. Our results suggest that intracellular localization of TDP-43 and proteasomal function may be involved in inclusion formation and neurodegeneration in TDP-43 proteinopathies.

1. Introduction

Frontotemporal lobar degeneration with ubiquitinated inclusions (FTLD-U) and amyotrophic lateral sclerosis (ALS) are well-known neurodegenerative disorders. FTLD is the second most common form of cortical dementia in the population below the age of 65 years [1]. ALS is the most common of the motor neuron diseases, being characterized by progressive weakness and muscular wasting, resulting in death within a few years. Ubiquitin (Ub)-positive inclusions were found as a pathological hallmark in brains of patients with FTLD-U and ALS, as well as in Alzheimer's disease (AD) and Parkinson's disease (PD). Recently, TAR DNA binding protein of 43 kDa (TDP-43) has been identified to be a major protein component of ubiquitin-positive inclusions in FTLD-U and ALS brains [2,3]. TDP-43 was first identified as a cellular factor that binds to the TAR DNA of HIV type 1 [4], and was also identified independently as part of a complex involved in inhibition of the splicing of the cystic fibrosis transmembrane conductance regulator (CFTR) gene [5]. TDP-43 aggregates in neuronal cytoplasm and nuclei in a variety of neurodegenerative disorders, which are now collectively referred to as TDP-43 proteinopathies. For understanding molecular pathogenesis and evidence-based therapies for TDP-43 proteinopathies, it is necessary to study the molecular mechanisms of aggregation of TDP-43.

To elucidate these issues, in this study, we have established the cellular models for intracellular aggregates of TDP-43 similar to those in brains of TDP-43 proteinopathies patients. Expression of deletion mutants of TDP-43 lacking two candidate sequences for the nuclear localization signal (NLS), residues 78–84 or 187–192, resulted in the formation of Ub- and phosphorylated TDP-43-positive cytoplasmic inclusions in the presence of a proteasome inhibitor. These results suggest that intracellular localization of TDP-43 and proteasomal function may be involved in the pathological process of TDP-43 proteinopathies.

Abbreviations: FTLD-U, frontotemporal lobar degeneration with ubiquitinated inclusions; ALS, amyotrophic lateral sclerosis; Ub, ubiquitin; TDP-43, TAR DNA binding protein of 43 kDa; CFTR, cystic fibrosis transmembrane conductance regulator; NLS, nuclear localization signal; TX, Triton X-100; Sar, Sarkosyl.

* Corresponding authors. Fax: +81 3 3329 8035 (T. Nonaka).
E-mail addresses: nonakat@prit.go.jp (T. Nonaka), masato@prit.go.jp (M. Hasegawa).

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2. Materials and methods

2.1. Construction of plasmids

The PCR product of the open-reading frame of human TDP-43 using pRc-CMV-TDP-43 as a template was subcloned into the mammalian expression vector pcDNA3 (Invitrogen) using restriction sites BamH I and Xba I, creating pcDNA3-TDP-43. To construct plasmids of the deletion mutants, we used a site-directed mutagenesis kit (Strategene). PCR was performed using the forward primer (5′-GTATGTTGTCAACTATATGGATGAGACAGATGC-3′) and the reverse primer (5′-GCATCTGTCTCATCCATATAGTTGACAACATAC-3′) for the deletion mutant of 78–84 residues (ΔNLS), and the forward primer (5′-GCAAAGCCAAGATGAGGTGTTTGTGGGGCGC-3′) and the reverse primer (5′-GCGCCCCACAAACACCTCATCTTGGCTTTGC-3′) for the deletion mutant of 187–192 residues (Δ187–192), with pcDNA3-TDP-43 as a template, respectively. For the construction of the double-deletion mutant ΔNLS&187–192, PCR was performed using the forward primer (5′-GCAAAGCCAAGATGAGGTGTTTGTGGGGCGC-3′) and the reverse primer (5′-GCGCCCCACAAACACCTCATCTTGGCTTTGC-3′) with pcDNA3-TDP-43ΔNLS as a template.

The reporter plasmid pSPL3-CFTR9 was constructed as follows. Healthy human genomic DNA (a gift from Dr. Makoto Arai, Tokyo Institute of Psychiatry, Japan) was subjected to PCR with the use of the forward primer (5′-CGGAATTCACTTGATAATGGGCAAATATC-3′) and the reverse primer (5′-CCCTCGAGCTCGCCATGTGCAAGATACAG-3′), containing EcoR I and Xho I sites, respectively. The genomic region containing 221 bp of intron 8, the entire exon 9 (183 bp), and 266 bp of intron 9 of the human CFTR gene was amplified and digested with the two restriction enzymes, followed by ligation into pSPL3 (Life Technologies), affording the plasmid pSPL3-CFTR9. All constructs were verified by DNA sequencing.

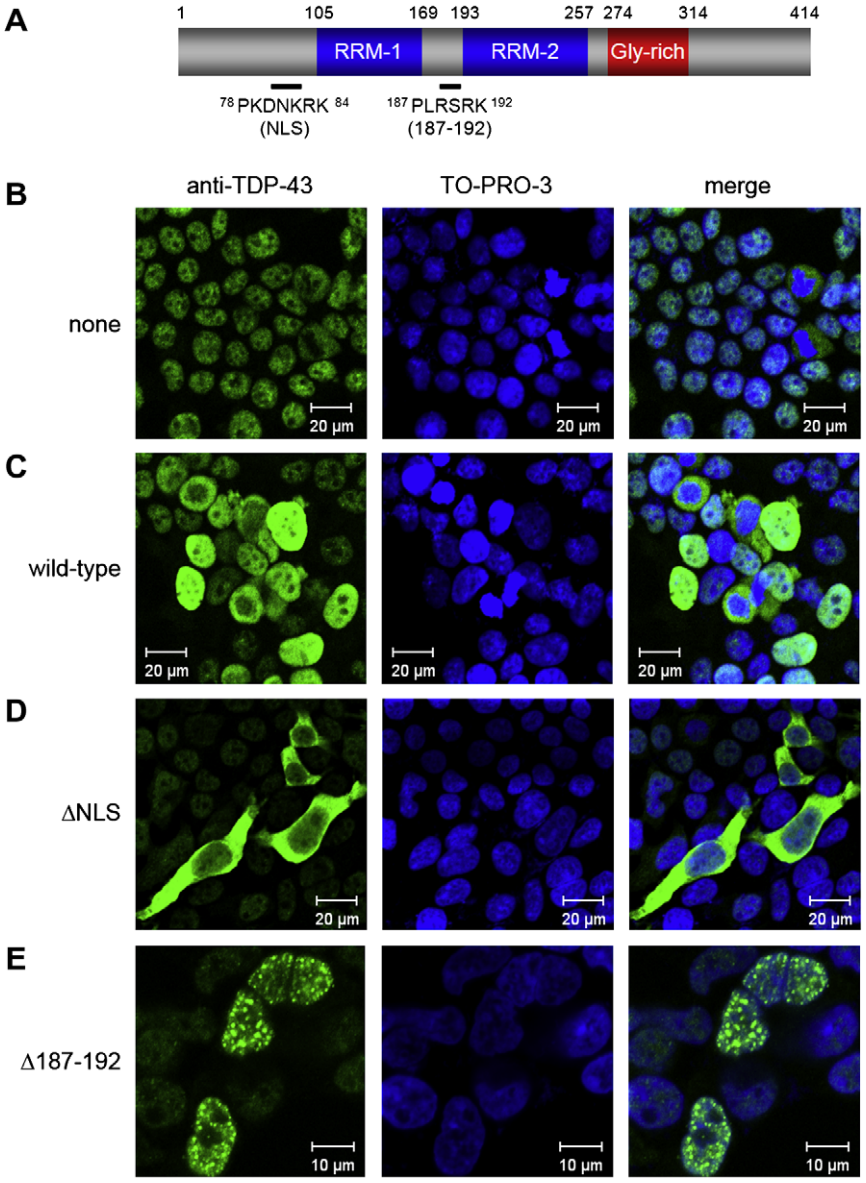

Fig. 1. Subcellular localization of wild–type and mutant TDP-43 in SH-SY5Y cells. (A) Schematic diagram of the structural domains of TDP-43. RNA recognition motifs (RRM-1 and -2: blue) and glycine-rich domain (Gly-rich: red) are shown. (B–E) Immunostaining of untransfected SH-SY5Y cells (B) and cells 72 h post transfection with wild-type TDP-43 (C), ΔNLS (Δ78–84) TDP-43 (D), and Δ187–192 TDP-43 (E) with anti-TDP-43 antibody (Left panel, green), nuclear staining by TO-PRO-3 (middle panel, blue) and the merged image (right panel) are shown.

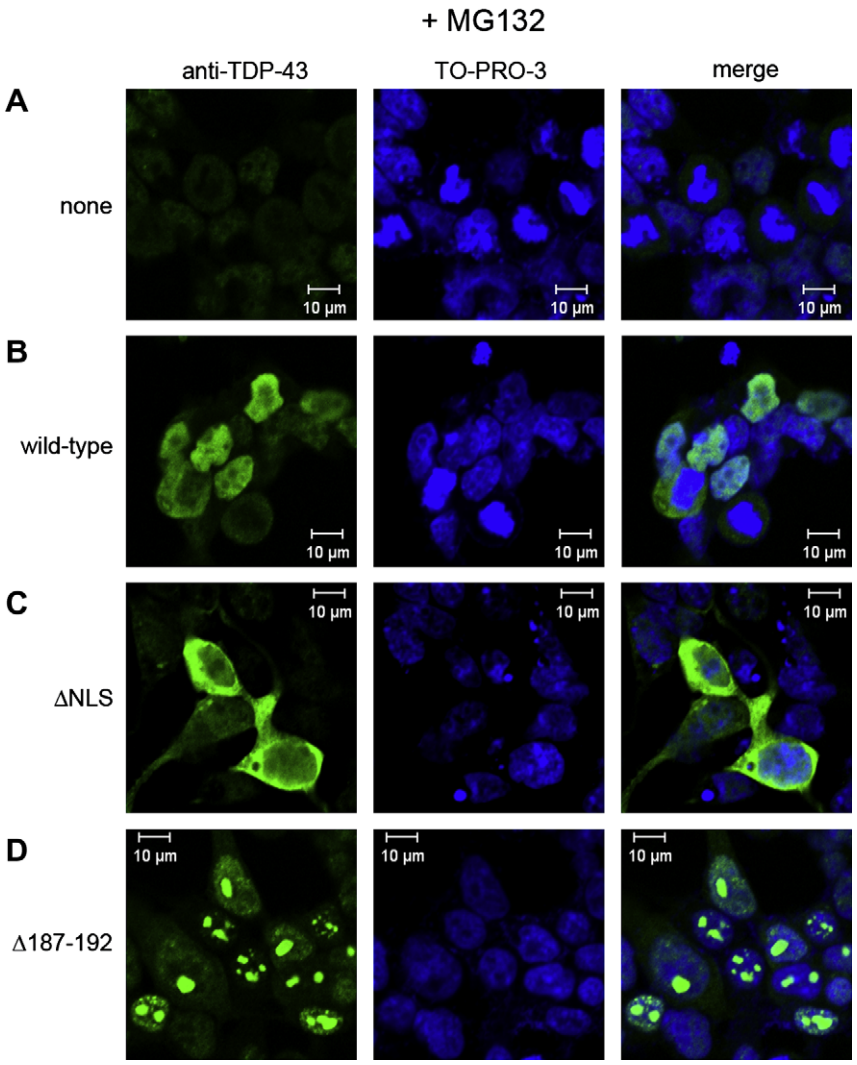

Fig. 2. Expression of mutant TDP-43 followed by proteasome inhibition with MG132 results in the formation of intranuclear inclusions. Immunostaining of untransfected cells (A) and cells 72 h post transfection with wild-type TDP-43 (B), ΔNLS (C), and Δ187–192 (D) followed by MG132 treatment (20 μM for 6 h) with anti-TDP-43 antibody (left panel, green), nuclear staining by TO-PRO-3 (middle panel, blue) and merged image (right panel).

2.2. Antibodies

A polyclonal TDP-43 antibody 10782-1-AP (anti-TDP-43) was purchased from ProteinTech Group Inc. A polyclonal antibody specific for phosphorylated TDP-43 (anti-pS409/410) was prepared as described [6]. Anti-ubiquitin monoclonal antibody (mAb), MAB1510, was purchased from Chemicon. Monoclonal anti-HA clone HA-7 were obtained from Sigma.

2.3. Cell culture and expression of plasmids

SH-SY5Y cells were cultured in DMEM/F12 medium (Sigma) supplemented with 10% (v/v) fetal calf serum, penicillin–streptomycin–glutamine (Gibco), and MEM non-essential amino acids solution (Gibco). Cells were then transfected with expression plasmids using FuGENE6 (Roche) according to the manufacturer's instructions. In the proteasome inhibition experiments, final 1 μM MG132 (Peptide institute) in DMSO was added to the culture medium, and incubated overnight.

2.4. Confocal immunofluorescence microscopy

SH-SY5Y cells were grown on a coverslip (15 × 15 mm) and transfected with expression vector (1 μg). After incubation for the indicated time, the transfected cells on the coverslips were fixed with 4% (w/v) paraformaldehyde in phosphate-buffered saline (PBS) for 30 min. The coverslips were then incubated with 0.2% (v/v) Triton X-100 (TX) in PBS for 10 min. After blocking for 30 min in 5% (w/v) BSA in PBS, cells were incubated with anti-phosphorylated TDP-43 antibody, pS409/410 (1:500 dilution), anti-TDP-43 (1:500), anti-Ub (1:500) or anti-HA (1:500) for 1 h at 37 °C, followed by FITC- or TRITC-labeled goat anti-rabbit or-mouse IgG (Sigma, 1:500 dilution) as a secondary antibody for 1 h at 37 °C. After washing, the cells were further incubated with TO-PRO-3 (Molecular Probes, 1:3000 dilution in PBS) for 1 h at 37 °C to stain nuclear DNA, and analyzed using a LSM5 Pascal confocal laser microscope (Carl Zeiss).

2.5. Sequential extraction of proteins and immunoblotting

SH-SY5Y cells were grown in 6-well plates and transfected transiently with expression plasmids (1 μg). After incubation for the indicated time, cells were harvested and lysed in TS buffer [50 mM Tris–HCl buffer, pH 7.5, 0.15 M NaCl, 5 mM ethylenediaminetetraacetic acid, 5 mM ethylene glycol bis(β-aminoethyl ether)-*N,N,N,N*-tetraacetic acid, and protease inhibitor cocktail (Roche)]. Lysates were centrifuged at 290,000×*g* for 20 min at 4 °C, and the supernatant was recovered as the TS-soluble fraction.

TS-insoluble pellets were lysed in TS buffer containing 1% (v/v) TX, and centrifuged at 290,000×*g* for 20 min at 4 °C. The supernatant was collected as TX-soluble fraction. TX-insoluble pellets were further sonicated in TS buffer containing 1% (w/v) Sarkosyl (Sar), and incubated for 30 min at 37 °C. The mixtures were centrifuged at 290,000×*g* for 20 min at room temperature, and the supernatant was recovered as the Sar-soluble fraction. The remaining pellets (insoluble in Sar) were lysed in SDS-sample buffer and heated for 5 min.

Each sample (10 or 20 μg) was separated by 12% (v/v) SDS–PAGE using Tris–glycine buffer system, and proteins were transferred onto polyvinylidene difluoride membrane (Millipore). The blots were incubated overnight with the indicated primary antibody at an appropriate dilution (1:1000–3000) at room temperature, followed by the incubation with a biotin-labeled secondary antibody. Signals were detected using an ABC staining kit (Vector).

2.6. CFTR exon 9 skipping assay

Cos-7 cells in 6-well plates were transfected with 0.5 μg of the reporter plasmid pSPL3-CFTR9 plus 1 μg of pcDNA3 plasmid encoding wild-type or its mutants, using FuGENE6. The cells were harvested 48 h post transfection, and total RNA was extracted with TRIzol (Invitrogen). The cDNA was synthesized from 1 μg of total RNA with the use of the Superscript II system (Invitrogen). Primary and secondary PCRs were carried out according to the instruction manual of the exon trapping system (Life Technologies).

3. Results

3.1. Effect of deletion of two candidate NLS in TDP-43

Amino acid sequence containing proline followed by a cluster of basic amino acids is known to be typical NLS. We found two such sequences in TDP-43, PKDNKRK (residues 78–84) and PLRSRK (residues 187–192) (Fig. 1A). To examine whether these sequences function as the NLS, we constructed corresponding deletion mutants of TDP-43 and expressed them in SH-SY5Y cells. We employed non-tagged TDP-43 plasmid for expression in cultured cells, since expression of hemagglutinin (HA)-tagged TDP-43 in SH-SY5Y cells caused formation of inclusion-like structures (Fig. S1), suggesting that addition of the epitope tag to the N-terminus may affect the conformation of TDP-43 and promote non-specific aggregate formation.

Endogenous TDP-43 expressed in the nucleus (Fig. 1B). Similar but stronger nuclear TDP-43 staining was observed in cells expressing wild-type TDP-43 (Fig. 1C), as compared with non-transfected cells (Fig. 1B). When the deletion mutant lacking residues 78–84 (ΔNLS) was transiently expressed, strong TDP-43 signals were detected in cytoplasm (Fig. 1D). This is reasonable because the sequence of 78–84 contains a part of the bipartite NLS in TDP-43 recently identified by Winton et al. [7].

When the deletion mutant lacking residues 187–192 (Δ187–192) was expressed, on the other hand, the mutant protein formed dot-like structures in nuclei (Fig. 1E). This observation suggests that this sequence does not function as a NLS.

3.2. Formation of intracellular TDP-43 inclusions in cultured SH-SY5Y cells

Impaired Ub–proteasome system has been suggested in some forms of neurodegenerative disease [8], and TDP-43 is indeed ubiquitinated in the brains of patients with FTLD-U or ALS [3]. To examine whether impaired Ub–proteasome system is involved in inclusion formation, we treated cells transfected with TDP-43 wild-type or deletion mutants with a proteasome inhibitor, MG132. In immunocytochemistry using phosphorylation-independent anti-TDP-43 antibody, no obvious change in the localization of TDP-43 was observed in mock cells (Fig. 2A), or in cells transfected with wild-type (Fig. 2B) or ΔNLS mutant (Fig. 2C) after MG132 treatment, as compared to those without MG132 (Fig. 1). In contrast, many round nuclear inclusions were generated in cells transfected with Δ187–192 mutant after MG132 treatment (Fig. 2D: ~12% of inclusion-positive cells).

Fig. 3 revealed the results of double immunostaining using anti-pS409/410, phosphorylation-dependent anti-TDP-43 antibody [6], and anti-Ub antibody. Cells expressing wild-type TDP-43 showed

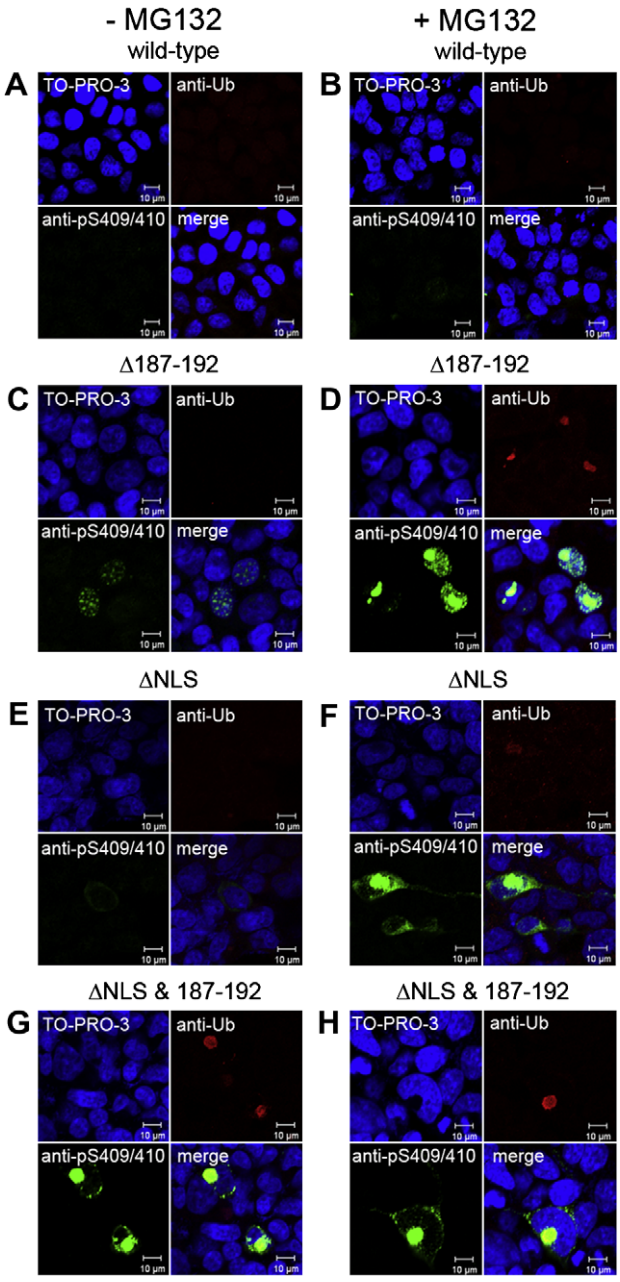

Fig. 3. Immunocytochemical analyses of intracellular inclusion-like structures formed in cells transfected with deletion mutants of TDP-43. SH-SY5Y cells 72 h post transfection with wild-type, (A and B), Δ187–192 (C and D), ΔNLS (E and F), and ΔNLS&187–192 (G and H) before (A, C, E, G) and after (B, D, F, H) treatment with MG132 (20 μM for 6 h) were stained with a phosphorylation-specific antibody, anti-pS409/410 (green), anti-ubiquitin (Ub: red) antibodies, and TO-PRO-3 (blue).

no immunoreactivity for anti-pS409/410 and anti-Ub antibodies in the presence or absence of MG132 (Fig. 3A and B). Cells transfected with Δ187–192 showed small dot-like structures positive for anti-pS409/410 antibody but negative for anti-Ub antibody without MG132 treatment (Fig. 3C). Cells transfected with Δ187–192 after the MG132 treatment contained inclusion-like structures in nuclei that were intensely labeled with both antibodies (Fig. 3D: ~8% of inclusion-positive cells). Relatively large inclusions (~5 μm) were immunopositive for both antibodies, while small ones (<1 μm) were positive for only phospho-specific TDP-43 antibody, suggesting that abnormal phosphorylation of TDP-43 is an early event in the process of inclusion formation.

Cells transfected with ΔNLS showed no immunoreactivity without MG132 treatment (Fig. 3E), but showed formation of cytoplasmic inclusions positive for both anti-pS409/410 and anti-Ub after MG132 treatment (Fig. 3F: ~10% of inclusion-positive cells). Furthermore, cells transfected with mutant TDP-43 lacking both the NLS and residues 187–192, named ΔNLS&187–192, formed intracellular round inclusion-like structures positive for both anti-pS409/410 and anti-Ub antibodies, independently of treatment

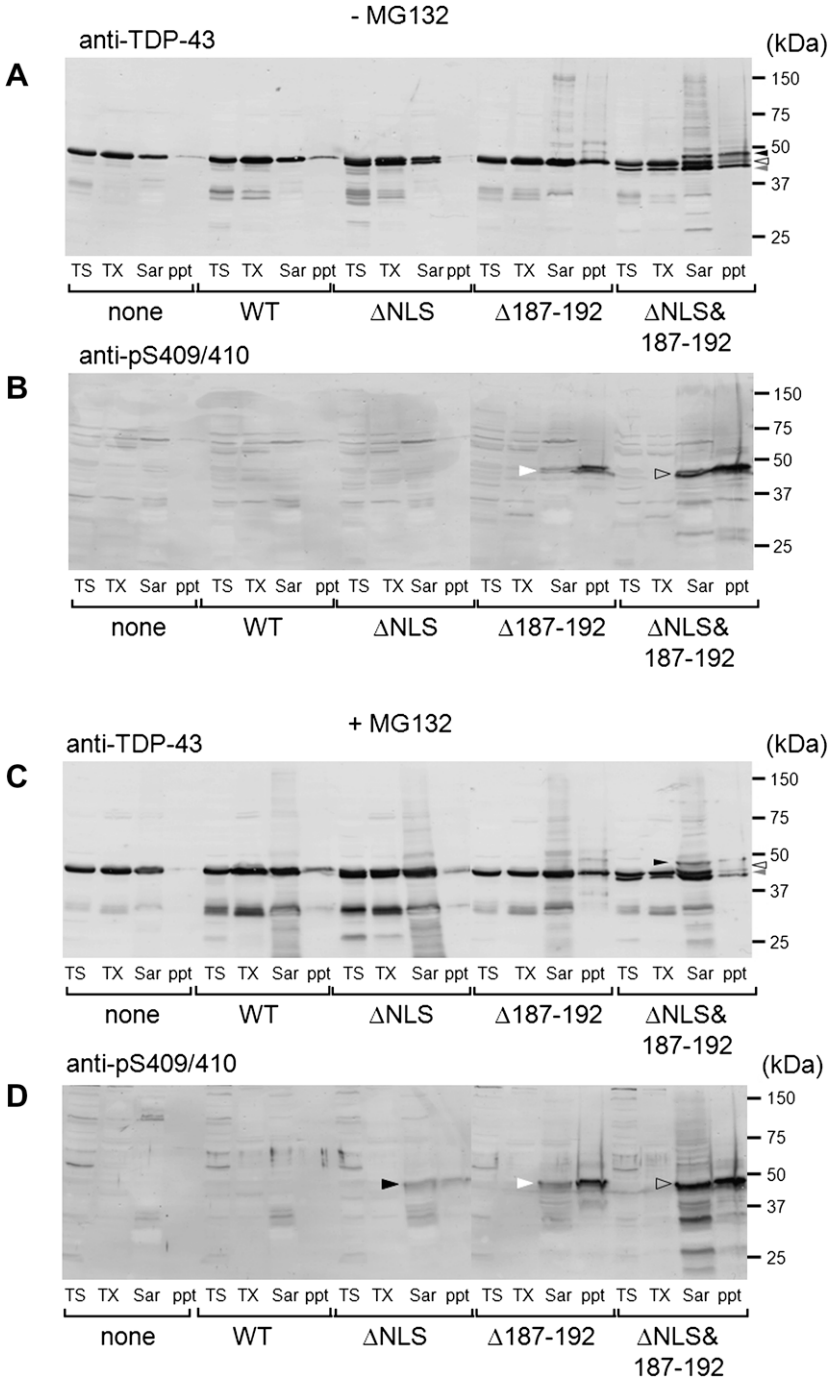

Fig. 4. Immunoblot analyses of intracellular inclusions of deleted TDP-43 mutants in SH-SY5Y cells. Untransfected cells (none) and cells 72 h post transfection with wild-type (WT) or TDP-43 deletion mutants, before (A and B) and after (C and D) treatment with MG132 (1 μM overnight), were sequentially extracted with Tris–saline (TS), 1% Triton-X (TX) and 1% Sarkosyl (Sar), and these supernatants and the Sarkosyl-insoluble pellets (ppt) were run on SDS–PAGE, transferred to PVDF membrane and probed with anti-TDP-43 antibody (A and C) and anti-pS409/410 (B and D).

with MG132 (Fig. 3G: ~8% of inclusion-positive cells and H: ~12% of inclusion-positive cells). The inclusions were ~10 μm in diameter, being very similar in size to the neuronal cytoplasmic inclusions found in patients with FTLD-U [2].

3.3. Immunoblot analysis of intracellular inclusions in the cultured cell models

Cells expressing wild-type or mutant TDP-43 were sequentially extracted, and the supernatants and pellets were analyzed by immunoblotting. On analysis of cell lysates using anti-TDP-43 antibody, endogenous TDP-43 at 43 kDa was detected in all fractions. Immunoreactivity of it was the strongest in TX-soluble fraction, and was the weakest in Sar-insoluble fraction (black-lined arrowheads in Fig. 4A and C). A similar band pattern but with stronger immunoreactivities was detected in cells transfected with wild-type TDP-43 (WT).

The ΔNLS mutant TDP-43, which was detected as bands with slightly lower molecular weight than that of the wild-type, was also recovered mostly in the TS- and TX-soluble fractions (Fig. 4A). The intensities of bands in the Sar-soluble and -insoluble fractions were slightly increased after MG132 treatment (Fig. 4C). Interestingly, these were positive for anti-pS409/410 (a black arrowhead in Fig. 4D), suggesting that inhibition of proteasome activity induces the aggregation of phosphorylated ΔNLS.

In contrast, expression of Δ187–192 mutant TDP-43, which was detected as a band with almost the same molecular weight as that of endogenous TDP-43, resulted in significant increases in the Sar-soluble and -insoluble (ppt) fractions as compared with those in cells transfected with wild-type TDP-43 (Fig. 4A). Smeared and higher-molecular-weight bands were also detected in the Sar-soluble and -insoluble fractions (Fig. 4A). We also observed pS409/410-positive bands in the Sar-soluble and -insoluble fractions in the absence or presence of MG132 (white arrowheads in Fig. 4B and D).

Finally, immunoblots of lysates from cells expressing ΔNLS&187–192 mutant TDP-43 (~41 kDa bands marked with grey arrowheads in Fig. 4A and C) showed high-molecular-weight bands of ~45 kDa (black arrowheads in Fig. 4A and C) and smears in the Sar-soluble and -insoluble fractions. The bands of ~45 kDa, smears, and C-terminal fragments at 25–37 kDa were highly immunoreactive with anti-pS409/410 antibody independently of MG132 treatment (black-lined arrowheads in Fig. 4B and D). These were similar characteristic band patterns to those found in immunoblot analyses of brain lysates of FTLD-U and ALS as previously reported [6].

3.4. Deletion mutants of TDP-43 lost the exon skipping activity

To evaluate the functional significance of the deletion mutants of TDP-43 used in this study, we performed CFTR exon 9 skipping assay. As shown in Fig. 5B, mRNA from cells transfected with empty vector pcDNA3 gave only one RT-PCR band of 360 bp, while that from cells transfected with pcDNA3-TDP-43 wild-type gave two RT-PCR bands, 360 and 177 bp, showing that skipping of CFTR exon 9 was increased by expression of wild-type TDP-43. In contrast, only one RT-PCR band of 360 bp was observed from cells co-transfected with ΔNLS, Δ187–192, or ΔNLS&187–192, indicating that these mutants do not have skipping activity of CFTR exon 9, which is one of known physiological functions of TDP-43.

4. Discussion

In this study, using SH-SY5Y cells and a phosphorylated TDP-43 specific antibody established by ourselves, we examined the effect of deletion of two candidate sequences for NLS, residues 78–84 and 187–192 of TDP-43, and proteasomal inhibition on inclusion formation. Mislocalization of TDP-43 into cytoplasm caused by deletion of residues 78–84 proves that this sequence indeed functions as NLS. This result is largely consistent with the previous report by Winton et al., which showed that residues 82–98 were

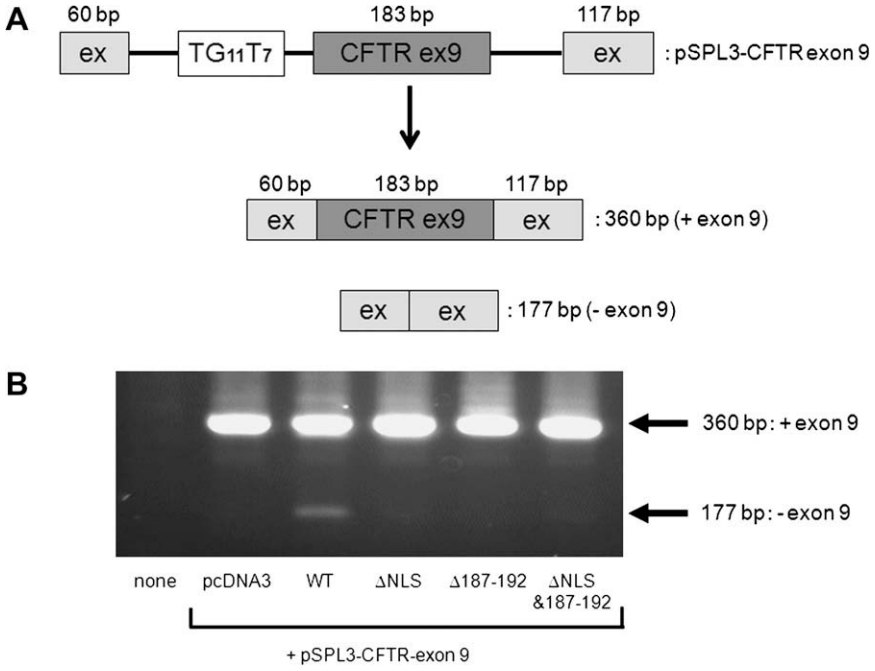

Fig. 5. CFTR exon 9 skipping assay of the deletion mutants of TDP-43. (A) Schematic diagram of the reporter plasmid pSPL3-CFTR exon 9. This plasmid contains the repeat sequence of TG11T7 in which the TG11 repeat is recognized by TDP-43, causing the CFTR exon 9 to be spliced out. The insert of this plasmid contains two exons of HIV-1 tat gene (60 and 117 bp, respectively: light grey boxes) flanking CFTR exon 9 (183 bp: a dark grey box). RT-PCR is expected to generate two products with (360 bp) and without CFTR exon 9 (177 bp). (B) Gel electrophoresis of RT-PCR products of RNA from transfected cos-7 cells. The RNAs from cos-7 cells, co-transfected with the reporter plasmid pSPL3-CFTR exon 9 plus pcDNA3 expression vectors were used as templates for RT-PCR analysis. The products were analyzed by electrophoresis in 1.5% agarose gel.

required for TDP-43 entry into the nucleus [7]. Formation of intranuclear TDP-43 positive dot-like structures caused by deletion of residues 187–192 suggests that this sequence does not function as a NLS but is nonetheless important to maintain a physiological state of TDP-43 in the nucleus. Loss of the exon skipping activity of CFTR exon 9 observed in cells transfected with this mutant may also support such a notion.

The results of the present study suggest that mislocalization of TDP-43 in cytoplasm is not a sufficient condition for aggregation of TDP-43, since the treatment of MG132, a proteasomal inhibitor, is needed to cause the formation of inclusion in cells transfected with a mutant TDP-43 lacking residues 78–84. Proteasome inhibition also induced formation of intranuclear inclusions in cells transfected with a mutant TDP-43 lacking residues 187–192. These results also suggest that a proteasome activity plays an important role for degradation of TDP-43. Impairment of the ubiquitin-proteasome system has recently been suggested to be related to the onset of neurodegenerative diseases. For instance, Bence et al reported that intracellular aggregates of a huntingtin fragment containing a pathogenic polyglutamine repeat directly impaired the function of the ubiquitin-proteasome system [9]. Keck et al., showed that proteasome was inhibited by paired helical filament-tau in brains of patients with Alzheimer's disease [10]. It should be further investigated whether the proteasome activity is actually decreased in brains of patients with TDP-43 proteinopathies, as Keller et al., reported that a significant decrease in proteasome activity was observed in AD brains [11]. Function of autophagy-lysosome degradation system may be an issue to be investigated as well, since inhibition of autophagic degradation by depletion of the endosomal sorting complexes required for transport (ESCRT) subunits causes accumulation of TDP-43 in ubiquitinated inclusions in cultured cells [12].

In contrast to a mutant lacking residues 78–84 or 187–192, double-deletion mutant of these sequences caused inclusion formation without proteasomal inhibition in this study. These results suggest the possibility that the double mutant protein has a higher propensity to aggregate than each single mutant protein. In this context, it should be noted that in insoluble fraction from FTLD-U brains, the amount of C-terminal fragments of TDP-43 is higher than that of the full-length TDP-43 [6]. These findings suggest that conformation or modifications of TDP-43 is another important factor for inclusion formation.

Importantly, intranuclear or cytoplasmic inclusions observed in this study were immunopositive for both a phosphorylation-dependent anti-TDP-43 antibody and anti-Ub antibody, suggesting that those consist of phosphorylated and ubiquitinated TDP-43. Biochemical analyses also support that abnormal phosphorylation of TDP-43 takes place in cells with inclusions. These results suggest that our cellular models recapitulate the phenotypes of TDP-43 proteinopathies both pathologically and biochemically. These models are expected to be valuable tools for understanding the pathological process underlying TDP-43 proteinopathies and for identifying candidate drugs to prevent intracellular aggregation of TDP-43.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.febslet.2008.12.031.

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